Solutes that Protect *Staphylococcus aureus* against Heat-Induced Injury and Their Effect on Cellular Leakage

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ABSTRACT

A number of compounds including salts, sugars, polyols, and amino acids protected *Staphylococcus aureus* 196E from injury during heating; additionally, most of the compounds reduced or prevented heat-induced leakage of 260 and 280 nm absorbing materials from the cells. In the presence of polyols (sorbitol, mannitol, xylitol, or glycerol) or fructose, heated cells leaked more ultraviolet-absorbing materials than controls lacking solutes even though the solute-treated cells showed little or no injury. The ability of polyols and fructose to induce leakage of cellular constituents during heating of *S. aureus,* even though few injured cells were found, suggests that leakage from cells undergoing stress does not always indicate injury.

Thermal injury and death in *Staphylococcus aureus* usually are accompanied by leakage of cellular constituents into the external medium. Allwood and Russell (*1*) showed a direct relationship between thermal death in *S. aureus* and leakage of cellular 260-nm absorbing materials. Membrane damage in thermally injured staphylococci was indicated by loss of cellular constituents including 260-nm absorbing materials, free amino acids, and potassium ions to the external medium (*11*). Since lipid in gram-positive bacteria is located only in the cell membrane (*12*), Hurst et al. (*9*) interpreted the leakage of lipid during sublethal heating as indicative of membrane damage. Hurst and his coworkers (*10*) also correlated leakage of magnesium ions during sublethal heating with the loss of staphylococcal salt tolerance (a measure of cellular injury). Thus, upon sublethal or lethal heat treatment, *S. aureus* may lose 260-nm absorbing materials, amino acids, lipids, and ions from the cells with such losses of cellular constituents probably indicating damage to the bacterial membrane.

Compounds which protect bacterial cells against lethal or injurious effects of heat appear to decrease or eliminate leakage of cellular constituents. Sucrose decreased heat lethality in *S. aureus* and heat injury in *Salmonella typhimurium;* in both organisms, there was a decrease in leakage of 260-nm absorbing materials (*1,13*). Little or no injury took place in *S. aureus* heated in the presence of NaCl and there was a marked decrease in leakage of cellular constituents (*15*). In this study, various compounds that protect *S. aureus* against heat injury were examined to determine their effect on leakage of ultraviolet absorbing materials from the cell.

MATERIALS AND METHODS

Preparation of cells

S. aureus 196E was inoculated into 100 ml of tryptic soy broth (Difco[2]) and incubated on a rotary shaker (200 rpm) at 35°C for 16 h. The contents of the culture flasks were centrifuged at 16,000 × *g* for 5 min at 5°C, washed three times with sterile potassium phosphate buffer (0.1 *M*, pH 7.2), and resuspended in 3 ml of sterile buffer.

Heat injury

All flasks, experimental and control, contained 100 ml of sterile phosphate buffer (0.1 *M*, pH 7.2) and washed cells of *S. aureus* at a final concentration of approximately 10^9/ml of buffer. Solutes were dissolved in 50 ml of sterile 0.2 *M* phosphate buffer and diluted to 100 ml with sterile distilled water. All flasks were equilibrated at 50°C in a constant temperature water bath and were continuously agitated during the experiment. Temperature was monitored with a thermocouple inserted below the surface of the heating menstruum. Temperature equilibration occurred approximately 3 min after addition of the cells.

One hundred microliter portions of each suspension were transferred to 9.9-ml sterile peptone (0.1%, Difco) water blanks after 0 and 45 min of heating to determine the extent of injury. Quantitation of injured and noninjured cells was achieved by differential plating onto tryptic soy agar + 1% sodium pyruvate (TSAP) and tryptic soy agar + 7% NaCl (TSAS) as previously described (*16*).

Determination of cell leakage

At the end of the 45-min incubation period at 50°C, 10 ml were removed from each injury and control flask, and cells

[1]*Agricultural Research Service, U.S. Department of Agriculture.*

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were removed by centrifugation (16,000 × *g*, 5°C, 5 min). Supernatant fluids were decanted and filtered through glass wool plugs into clean tubes. Ultraviolet (UV) absorbing materials were determined using a Hewlett-Packard UV/VIS Spectrophotometer (Model 8450 A). Distilled water was used as the blank for the UV determinations. The values obtained from the inoculated phosphate buffer with or without solute were corrected for background solute absorption by substracting the uninoculated controls.

Orcinol and diphenylamine tests

The orcinol and diphenylamine tests for pentose and deoxypentose sugars, respectively, were those of Ashwell (*3*).

Source of chemicals

The inorganic salts, glucose, and glycerol were obtained from J. T. Baker Chemical Company, Phillipsburg, NJ. Sugars (including α-methylglucoside) other than glucose, polyols other than glycerol, sodium pyruvate, and the amino acids were obtained from Sigma Chemical Company, St. Louis, MO.

RESULTS

All solutes tested in the present study protected *S. aureus* against the injurious effects of heat (Table 1). When cells were heated in phosphate buffer containing salts, sugars, polyols, or amino acids, most compounds reduced the injury incurred by the cells to 5% or less of that found in controls lacking solute. The greatest amount of injury was noted with mannitol and fructose but that was only about 10% of control values.

Cells lost more 260- and 280-nm absorbing materials to the medium when heated in buffer containing polyols (sorbitol, mannitol, xylitol, or glycerol) or fructose than in buffer alone (Table 1). This excessive leakage occurred even though the amount of injury in solute buffer was less than 10% of the controls. In the presence of the other solutes, staphylococci leaked ≤40% of the UV absorbing materials as compared to the controls (Table 1). Therefore, most of the compounds that prevented heat injury also reduced or prevented heat-induced cellular leakage. Leakage of 260- and 280-nm absorbing materials was not found in the control flasks (lacking solute) incubated for 45 min at 2°C; however, leakage did occur at 36°C but it was only about one-third of that found at 50°C. The polyols or fuctose did not stimulate leakage at 2 or 36°C as they did at 50°C.

When the cells were removed from the heating menstruum and the supernatant fluid subjected to the orcinol and diphenylamine tests, only the controls and solutions containing mannitol, xylitol, and glycerol showed positive tests with the orcinol reagent (sorbitol and fructose gave brown precipitates), suggesting that ribose-containing materials had leaked from the cells. All of the polyol-containing supernatant fluids and the controls gave a negative reaction of diphenylamine reagent (fructose gave a brown precipitate), thereby suggesting that deoxyribose-containing materials did not leak from the cells. The significance of the brown precipitates obtained with sorbitol (orcinol tests) and with fructose (orcinol and diphenylamine tests) is not known.

The A_{280}/A_{260} ratio of the control (lacking solutes) after removal of injured cells was 0.475 (mean of 10 experiments), which is suggestive of leakage of both nucleic acids and nucleoproteins (*11*).

The water activity (a_w; calculated) of nine of the compounds in solution was 0.900 (Table 1). Seven of these

TABLE 1. *Comparison of the effect of solutes on heat injury and leakage of ultraviolet absorbing materials in* Staphylococcus aureus *196E.*

Additions	Concentration of added solute, *M*	Calculated a_w[a]	Log number of injured cells[b]	A_{260}[c]	% of control[d]	A_{280}[c]	% of control[d]
None	0.0	-	4.00	2.000	100	1.000	100
NaCl	2.7	0.900	0.07	0.340	17	0.294	29
KCl	2.8	0.900	0.12	0.000	0	0.000	0
NH_4Cl	2.7	0.900	0.00	0.169	9	0.089	9
Sorbitol	2.7	0.900	0.00	2.235	112	1.479	148
Mannitol	1.1	-	0.42	3.190	160	1.120	112
Xylitol	3.3	-	0.18	3.157	158	1.321	132
Glycerol	3.7	0.900	0.07	3.219	161	1.442	144
Sucrose	1.7	0.900	0.00	0.087	4	0.176	18
Maltose•H_2O	1.7	0.900	0.00	0.320	16	0.404	40
Glucose	2.7	0.900	0.00	0.033	2	0.128	13
Fructose	2.8	-	0.41	4.666	233	4.336	434
α-Methylglucoside	2.6	-	0.08	0.736	37	0.366	37
β-Alanine	3.5	0.900	0.20	0.387	19	0.209	21
L-Arginine•HCl	1.5	-	0.00	0.218	11	0.201	20

[a] a_w = water activity; a_w of the salts was calculated by the method given by Troller and Christian (*17*) and the a_w of polyols, sugars, and amino acids was calculated by the methods given by Chirife et al. (*6,7*); a dash (-) indicates that a_w was not determined.

[b] Log number of injured cells was calculated by (log $TSAP_{45\ min}$-$TSAS_{45\ min}$). The experimental data were normalized to the control which was arbitrarily set at log number of injured cells = 4.00.

[c] The 280/260 ratio averaged 0.475 for all of the controls. The A_{260} of the control was arbitrarily set at 2.000 and the A_{280} of the control was set at 1.000. All experimental data were normalized to the control.

[d] The controls were arbitrarily set at 100% and experiments were compared to the controls.

compounds (NaCl, KCl, NH_4Cl, sucrose, maltose•H_2O, glucose and β-alanine) did not induce excessive leakage, whereas sorbitol and glycerol produced more leakage than the control (Table 1). Since the a_w was similar in all of these compounds, it appears that excessive leakage of UV absorbing materials is not a function of lowered a_w.

DISCUSSION

Certain solutes can decrease or prevent injury and/or death if present during heating of the bacterial cells. The extent of death and leakage of 260-nm absorbing materials was decreased in the presence of 1 *M* sucrose during the heating of *S. aureus (1)*. Death and injury of *S. typhimurium* during heating has also been reported to be decreased when 0.88 *M* sucrose was present in the heating medium (*13*). Additionally, there was a reduction in the loss of 260-nm absorbing materials from the cells. Smith et al. (*15*) have shown that in the presence of 0.85 *M* NaCl, little or no injury took place when *S. aureus* was heated and the salt prevented leakage of 260-nm absorbing materials and decreased the extent of Mg^{++} loss.

Data obtained in the present study indicate that most compounds that protect *S. aureus* against heat injury also reduce the extent of leakage of 260-nm absorbing materials from the cells. However, extensive release of UV-absorbing materials occurred with polyols or fructose, although the cells only showed slight injury. The mechanism by which polyols or fructose lead to heat-induced leakage without producing injury is unknown but various factors can be eliminated. Water activity, ionic strength, osmolality, or ionic effects can be eliminated as bases for injury protection (*15,16*) and probably leakage induction.

The carbonyl group appears not to be critical since both sucrose and α-methylglucoside protect against injury and do not induce leakage. The principal difference between polyols and their respective carbonyl-bearing carbohydrates is the acylic configuration of the polyols in contrast to the ring configuration of the carbohydrates (*14*). While fructose exists mostly in the ring configuration (*2,4*), it can convert to the straight chain form more readily than glucose (ca. 250 times). Therefore, the ease in conversion of fructose to the straight chain conformation may allow it to induce leakage in *S. aureus* similarly to that of the acylic polyols. When fructose is constrained in the ring form, as in sucrose, then leakage of the cellular constituents would not occur.

Heat-injury is routinely accompanied by degradation of ribosomal RNA, as well as leakage of cellular constituents into the external medium (*5,8,18*). Leakage indicates that a lesion has been produced in the semi-permeable membrane, which allows RNA decomposition products, as well as other constituents, to leak from the cell. In the present experiments, polyols and fructose prevented heat injury but allowed leakage; thus, leakage can occur in the absence of injury. Heat-injury may always be accompanied by leakage of UV-absorbing materials; however, leakage may not always be indicative of injury.

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